OLIGONUCLEOTIDE FOR GENOTYPING *MYCOPLASMA* AND ITS RELATED STRAINS, MICROARRAY COMPRISING THE OLIGONUCLEOTIDE, AND METHOD FOR DETECTING STRAINS USING THE MICROARRAY

Technical Field

The present invention relates to a method for detecting *Mycoplasma* and its related strains which are a source of contamination of cell lines and biological products and human pathogens. More particularly, the present invention relates to genus-specific and species-specific oligonucleotides for genotyping *Mycoplasma*, *Acholeplasm* and *Ureaplasma* strains, a microarray comprising the oligonucleotides, and a method for detecting strains using the microarray

Background Art

Mycoplasma is a prokaryote pertaining to Mollicute family without cell wall, which was known as a hospital acquired pathogen causing pneumonia via infection of genital and respiratory organs of human as well as livestock such as pig and cow. Recently, *Mycoplasma* is more seriously understood as a major contaminant of cell culture and cell line

Especially, as the development and production of biological products for protecting and treating human diseases increases, the contamination of various pathogens provided by microorganism or clinical sample in the process of production became a serious problem. Examples of the biological products are an oncolytic virus, vaccine, a gene therapy vector and a recombinant protein. They have been found to be contaminated by bacteria, fungus, virus, *Mycoplasma* and its related strains (Doblhoff-Dier et al., 2001). The reason of the contamination is an organism contaminated in media components or experimental instruments and cross-contamination of microorganism and virus in air (Jung et al., 2003). Also, the contamination can be occurred

by a cross-contamination of already-infected WCB (Working Cell Bank) which is used for mass production of biological products (Wisher et al., 2002).

It is reported that, among these contamination sources, about 15~35% of cell culture or cell line is infected by *Mycoplasma* and its related strains (Hopert et al., 1993). This also makes experimental results incredible because it can change characteristics of cells such as abnormal synthesis of DNA, RNA and protein by binding to host cell wall (Kong et al., 2001). As gene therapy and cell therapy are getting into the spotlight recently, an assay for infection of stem cell and cord blood by *Mycoplasma* and its related strain became more important. Therefore, for the credible and reproducible experimental results and the quality control of commercialized biological products, it is essential to detect an infection with Mytcoplasma and its related strains.

Under this situation, Europe community make it a rule that, for credibility of safety and quality of food and drug, GMP (Good Manufacturing Practice) and QC (Quality Control) should be submitted and cell banks such as MCB (Master Cell Bank) and WCB should be subjected to an assay for detection of virus, fungus and bacteria such as *Mycoplasma* (Doblhoff-Dier et al., 2001).

About 100 kinds of bacteria pertaining to Mollicute family without cell wall have been found so far, including *Acholeplasma, Enteroplasma, Mesoplasma, Mycoplasma, Ureaplasma* and *Spiroplasma*. Among them, about 20 kinds of *Mycoplasma*, *Acholeplasma* and *Ureaplasma* are major contamination source of cell culture. These are referred to as "*Mycoplasma* and its related strains" in this specification. About 95% of the contaminants are covered by *M. arginini*, *M. fermentans*, *M. orale*, *M. hyorhinis*, *M. hominis*, *M. salivarium*, *M. pirum*, *A. laidlawii* (Dorigo-zetsma et al., 1997). However, *Mycoplasma* is difficult to be cultured in extracellular media and turbidity is rare in the culture. Therefore, there has been a need to the rapid and accurate genotypic detection method

which can trace a contamination source of *Mycoplasma* and its related strains.

Conventional *Mycoplasma* detection methods are the culturing method, the DNA fluorochome stain method, the immunofluorescence method, and the polymerase chain reaction (PCR) method (Dorigo-zetsma et al., 1997). However, the culturing method has a drawback that extracellular culturing is difficult, preparing its media is complex by adding supplements such as serum and culturing time is too long, about 4 days ~ 3 weeks according to the kinds of strains (Jensen et al., 2003). The DNA fluorochome stain method such as Hoechest 33258 stain has a drawback that culturing condition is too difficult to match and subjective inspectors can make a misjudgment (Chen et al., 1997). The immunofluorescence method such as ELISA has a drawback that bacteria having similar antigen with *Mycoplasma* such as *Streptococcus milleri group* and *Staphylococcus aureus* may raise a false positive signal due to of low specificity (Hopert et al., 1993). The PCR method makes use of 16S/23S intergenic spacer region (ITS) and a gene coding 169 kDa of P1 cyadhesion proteine which represent variety of *Mycoplasma* (Uphoff et al., 2002). The P1 gene, a surface antigen gene, has several subtypes representing diversity and has been used as a target gene for serological detection using immune reaction and genotypic detection using restriction fragment length polymorphism (RFLP) to identify *Mycoplasma* (Campo et al., 1998). However, most of conventional PCR methods use a primer designed based on 16S rRNA which is a common sequence of prokaryotes, and second PCR or nested PCR having high sensitivity can make a cross-contamination of *Mycoplasma* dispersed in air and an amplification of a bacteria similar with *Mycoplasma* in classification (Uphoff et al., 2002).

To overcome the above limitations of the conventional detection methods, a genotypic detection method using probes have been developed recently, which make it possible to analyze many kinds of

genes in a short time using DNA hybridization principle based on gene sequencing and detect specifically a single base change using a proper hybridization condition between specific probe and target DNA.

The present inventors developed ITS-derived oligonucleotides capable of detecting *Mycoplasma* and its related strains, which are important in genotypic detection, and a mycroarray comprising the oligonucleotides as a probe for detecting *Mycoplasma* and its related strains.

Disclosure of the Invention

It is a first object of the present invention to provide oligonucleotides for detecting *Mycoplasma* and its related strains designed based on their ITS base sequences.

It is another object of the present invention to provide novel ITS sequences of *Mycoplasma bovis*, *Mycoplasma cloacale*, *Mycoplasma falconis*, *Mycoplasma faucium*, *Mycoplasma spermatophilum* and *Mycplasma synoviae*, which is useful for detecting *Mycoplasma* and its related strains.

It is another object of the present invention to provide a microarray comprising genus-specific and species-specific oligonucleotides for detecting *Mycoplasma* and its related strains as probes.

It is another object of the present invention to provide a method for detecting *Mycoplasma* and its related strains using the microarray.

It is another object of the present invention to provide a kit for diagnosing *Mycoplasma* and its related species infection individually or simultaneously, comprising genus-specific and species-specific oligonucleotides for genotyping *Acholeplasma* and related strains.

According to an aspect of the present invention, there is provided a purified ITS (internal transcribed spacer) target DNA for genotyping

Mycoplasma strains, comprising any one sequence selected from SEQ ID Nos. 1 to 6.

SIQ ID Nos. 1 to 6 are base sequences of ITS (internal transcribed spacer) of *Mycoplasma bovis*, *Mycoplasma cloacale*, *Mycoplasma falconis*, *Mycoplasma faucium*, *Mycoplasma spermatophilum* and *Mycplasma synoviae*, which was newly obtained by base sequencing analysis.

The ITS target DNA of the present invention can be used indirectly for designing probes or primers used for genotyping *Mycoplasma* strains or directly for genotyping *Mycoplasma* strains via PCR amplification.

According to another aspect of the present invention, there is provided an oligonucleotide for genus-specific genotyping of *Mycoplasma* and *Ureaplasma* strains, comprising any one sequence selected from SEQ ID Nos. 7 to 21 or its complementary sequence.

According to another aspect of the present invention, there is provided an oligonucleotide for genus-specific genotyping of *Acholeplasma* strains, comprising any one sequence selected from SEQ ID Nos. 22 to 27 or its complementary sequence.

According to another aspect of the present invention, there is provided an oligonucleotide for species-specific genotyping of *Mycoplasma* and *Ureaplasma* strains, comprising any one sequence selected from SEQ ID Nos. 28 to 127 or its complementary sequence.

According to another aspect of the present invention, there is provided an oligonucleotide for species-specific genotyping of *Acholeplasma* strains, comprising any one sequence selected from SEQ ID Nos. 128 to 133 or its complementary sequence.

The oligonucleotides according to the present invention are designed based on multiple sequence alignment of ITS (internal transcribed spacer) sequences, which are present between 16S rRNA and 23S rRNA of *Mycoplasma* and its related species. The

oligonucleotides can be used as primers for PCR amplification in order to genotype *Mycoplasma* and its related species or as probes for hybridization reaction in order to genotype *Mycoplasma* and its related species.

According to another aspect of the present invention, there is provided a microarray comprising more than one oligonucleotides selected from genus-specific and species-specific oligonucleotides for genotyping *Mycoplasma*, *Acholeplasma* and *Ureaplasma* strains according to any one from claims 2 to 5 as probes attached on a support.

In the microarray according to the present invention, the probes may be any materials having base sequence, preferably any one selected from a group consisting of DNA (Deoxyribose Nucleic acid), RNA (Ribose Nucleic Acid), and nucleic acid analogues such as PNA (Peptide Nucleic Acid), LNA (Locked Nucleic Acid) and HNA (Hexitol Nucleic Acid).

In the microarray according to the present invention, the support may be any materials to which the probes can be attached, preferably any one selected from a group consisting of slide glass, plastic, membrane, semiconductive chip, silicon and gel. The microarray according to the present invention can be manufactured using conventional method such as pin microarray, ink jet, photolithography or electric array method.

The microarray according to the present invention can be used for simultaneously genotyping various *Mycoplasma* and its related species which are known as a major contaminant of biological drug and cell line as well as a human pathogen from one sample, as the microarray comprises genus-specific and species-specific oligonucleotides for genotyping *Mycoplasma* and its related species as a set attached a support

According to another aspect of the present invention, there is provided a method for detecting *Mycoplasma*, *Acholeplasma* and

Ureaplasma strains, comprising the following steps:

a) extracting nucleic acids from a sample;

b) amplifying target DNA among the extracted nucleic acids;

c) hybridizing the amplified target DNA with probes of the microarray according to the above present invention; and

d) detecting signals generated from the hybridization reaction.

In the detection method according to the present invention, the sample may be biological drug, cell line, or human tissues or serum. The purifying step can be performed using conventional DNA or RNA purification method or kit. The signal detecting step can be performed using a conventional fluorescence scanner after binding conventional fluorescent dyes such as Cy5 or Cy3.

According to another aspect of the present invention, there is provided a kit for diagnosing *Mycoplasma* and its related species infection, comprising more than one oligonucleotide selected from genus-specific and species-specific oligonucleotides for genotyping *Acholeplasma*, *Mycoplasma* and *Ureaplasma* strains according to the above present invention.

In the kit according to the present invention, the oligonucleotides are used as probes for hybridizing with target sample and may be contained in a proper vessel. The probes may be labeled with a radioactive or non-radioactive labeling agent, the latter comprises conventional biotin, Dig(digoxigenin), FRET(fluorescence resonance energy transfer) or fluorescent dye (Cy5 or Cy3). Further, the oligonucleotides can be used as primers for PCR amplification. In this case, the kit may contain DNA polymerase, 4 dNTPs and PCR buffer for PCR reaction. In addition, the oligonucleotides can be attached to a microarray as probes. In this case, the kit may contain hybridization reaction buffer, PCR kit containing primers for amplifying a target gene, washing solution for the unhybridized DNA, dyes, washing solution for unbound dyes and manual sheet for the microarray.

Hereafter, the present invention will be described in more detail.

The present invention provides a method for detecting or genotyping *Mycoplasma* and its related strains which is a major contamination source of cell lines and biological products and a human pathogen, comprising the following steps:

a) if necessary, extracting nucleic acids from a sample such as cell lines, biological products or human tissue or serum;

b) if necessary, amplifying target DNA of *Acholeplasma*, *Mycoplasma* and *Ureaplasma* strains among the extracted nucleic acids using more than one proper primers;

c) hybridizing the amplified target DNA with probes having a sense or antisense or complementary sequences of genus-specific and species-specific oligonucleotides of *Acholeplasma*, *Mycoplasma* and *Ureaplasma* strains disclosed in Tables 2 and 3; and

d) detecting signals generated from the hybridization reaction.

From the detected signals in the step d), the existence of *Mycoplasma* and its related strains in the sample can be predicted.

The present inventors carried out a sequence analysis of ITS regions of many *Acholeplasma*, *Mycoplasma* and *Ureaplasma* strains to obtain genus-specific and species-specific oligonucleotides for detecting Mycoplasma and its related stains which can be a basis of developing a specific and sensitive hybridization assay. Also, the present inventors newly analyzed ITS sequences of newly found 6 Mycoplasma strains, which makes it possible to design probes capable of detecting more various *Mycoplasma* and its related strains.

Table 1 discloses ITS sequences of newly analyzed 6 strains among target sequences for detecting *Mycoplasma* strains, which correspond to SEQ ID Nos. 1 to 6. In the present invention, the probes for detecting *Mycoplasma* strains were designed based on the multiple alignment of ITS sequences of *Mycoplasma*.

FIGS. 1 and 2 show multiple sequence alignments of ITS regions

of *Mycoplasma*, *Acholeplasma* and *Ureaplasma* for selecting genus-specific and species-specific probes of *Mycoplasma* and its related strains. Genus-specific oligonucleotides of *Mycoplasma* and *Ureaplasma* were designed from conservative sequence region indicated by a box in FIGS. 1a to 1f. Species-specific oligonucleotides of *Mycoplasma* and *Ureaplasma* were designed from polymorphic sequence region outside the box in FIGS. 1a to 1f. Genus-specific oligonucleotides of *Acholeplasma* were designed from conservative sequence region indicated by a box in FIGS. 2a to 2c. Species-specific oligonucleotides of *Acholeplasma* were designed from polymorphic sequence region outside the box in FIGS. 2a to 2c.

In step b) of the present invention, the target DNA of *Acholeplasma*, *Mycoplasma* and *Ureaplasma* strains were amplified using more than one pair of proper primers. FIG. 3 shows PCR amplification of ITS target sequences of *Mycoplasma* and its related strains using a primer pair, MP16SF-2 and MP23SR-2. In FIG. 3, 1 is a PCR product of *M. arginini*, 2 is a PCR product of *M. arthritidis*, 3 is a PCR product of *M. fermentans*, 4 is a PCR product of *M. hominis,* 5 is a PCR product of *M. hyorhinis*, 6 is a PCR product of *M. neurolyticum*, 7 is a PCR product of *M. opalescens*, 8 is a PCR product of *M. orale*, 9 is a PCR product of *M. pirum*, 10 is a PCR product of *M. penetrans*, 11 is a PCR product of *M. pulmonis*, 12 is a PCR product of *M. salivarium*, 13 is a PCR product of *M. cloacale*, 14 is a PCR product of *M. falconis*, 15 is a PCR product of *M. faucium*, 16 is a PCR product of *M. hyosynoviae*, 17 is a PCR product of *M. muris*, 18 is a PCR product of *M. primatum*, 19 is a PCR product of *M. spermatophilum*, 20 is a PCR product of *M. synoviae*, 21 is a PCR product of *M. pneumoniae*, 22 is a PCR product of *M. genitalium*, 23 is a PCR product of *M. bovis*, 24 is a PCR product of *U. urealyticum*, 25 is a PCR product of *A. laidlawii*.

In step c) of the present invention, the amplified target DNA were hybridized with probes for detecting *Mycoplasma* and its related strains.

Preferably, the probes may be a combination of more than one probes capable of simultaneously detecting many *Mycoplasma* and its related strains from a single sample. Practically, the probes are optimized to simultaneously hybridize with multiple target DNAs of *Mycoplasma* and its related strains under the same hybridization and washing conditions.

The present invention provides a microarray comprising a set of probes for detecting *Mycoplasma* and its related strains, which can simultaneously detect many *Mycoplasma* and its related strains from a single sample with a single experiment.

In the present invention, the term 'probe' means a single-stranded oligonucleotide having a sequence complementary to target DNA of *Mycoplasma*, *Acholeplasma* and *Ureaplasma*. The probe may have a sense, antisense or complementary sequence of SEQ ID Nos. disclosed in this specification as long as it can hybridize with one of double strands of target DNA. The oligonucleotide may be ribonucleotide (RNA), deoxynucleotide (DNA), peptide nucleic acid (PNA) or locked nucleic acid (LNA), and contain modified nucleotides such as Inosine only if it does not change their hybridization characteristics. Preferably, the genus-specific oligonucleotides for detecting *Mycoplasma*, *Acholeplasma* and *Ureaplasma* may have a base sequence of SEQ ID Nos. 7 to 27. Preferably, the species-specific oligonucleotides for detecting *Mycoplasma*, *Acholeplasma* and *Ureaplasma* may have a base sequence of SEQ ID Nos. 28 to 133.

FIG. 4 shows a microarray comprising probes for detecting genotypes of *Mycoplasma* and its related strains as a set on a support. In FIG. 4, each species name and SEQ IN Nos. are described which correspond to individual probes. The terms 'MP-C' and 'AP-C' mean *Mycoplasma* and *Ureaplasma* genus and *Acholeplasma* genus. FIG. 4 is no more than an example of probe compartment of the present invention, so compartment and layout of each probe can be varied.

In the present invention, newly analyzed ITS sequences of 6

Mycoplasma strains as a target DNA for detecting *Mycoplasma* and its related strains are as shown in Table 1. The genus-specific oligonucleotides for detecting *Mycoplasma*, *Acholeplasma* and *Ureaplasma* used in the present invention are as shown in Table 2. The species-specific oligonucleotides for detecting *Mycoplasma*, *Acholeplasma* and *Ureaplasma* used in the present invention are as shown in Table 3.

【Table 1】

Species	Sequence (5' → 3')	SEQ ID NO.
M.bovis	TTCTACGGAGTACACTTGTCTTTTATCACTATAAAAAAAAGACTTATAACCAAAAT TACTAGACCTATATTTATTTATAAACGTCATGGCTTTTATTAATAGGTCAAAAGCTA TATATCTAGTTTTGAGAGAACATTCTCTCATATGTTCTTTGAAAACTGAATAGTAAA ATATTTTTCGATATTTACAACGACATCAAAAATCAAATTAATGGTTAATTTGTTTTG ATTCATCGAGTAAGTCATATTTAATATGATTCATTGAAATGTCTTAAAATACACATC TAAAACTAACAACAATAGGAAAATACTACTTTTAAATAAGGAAGAGTTTTTGGTGG ATGC	1
M.cloacale	CTTCTACGGAGTACAATTCTCACTGTTATGGAATTAAATTTGTATCCAGTTTTGAGA GAACTTTCTCTCAATTTTGTTCTTTGAAAACTGAATATAGACATTGAAATCAATAAA TTAATATTTCAAATGTTTAGATCAACCTATAGAATATTCAAGACATATACAAAAATA GGTCATACTTATATTTATAAATACT	2
M.falconis	CTTTCTACGGAGTACAACTTCTGTTATGGAATAATATTTGTATCCAGTTTTGAGAGT ACTAACTCTCTTTTTGTTCTTTGAAAACTGAATATCGACATTGAAAAATTATTAATT AATATTTCAAAGTTTAGATCAACCTATAGAATACAAAAATATAGACAACAATAGGT CATACAACAAACATAACAAAACAACT	3
M.faucium	GAATGGTGGCTTCGAGACTAAAAGTTATGGAAAAACATCGTATCCAGTTTTGAGA GAACTAAACTTCTCTCTTTTGTTCTTTGAAAACTGAATATAGACATTGAAAATTAAA AAATTAATATTTCAAAGTTTAGATCAACCTATAGAATACAAAATCAATACAATAGG TCAATACTATACAATTGCATAACAAAAAATACTATTAAACAAGATAAGAGTTTTTG GTGGATGCAATTGTA	4
M.spermatophilum	GTGGGGATGGATCACCTCCTTTCTACGGAGTACAAACATACATTCAAATTTTGACT GAATGTTATTAACCTTATTTTTCACTAGGCCTTTTTAATATATTTTGTTATGTGACT TTTATGGCCTAAAAGTCTTATATCTAGTTTTGAGAGGACATCCTCTCTAATTGTTCT TTGAAAACTGAATAGTAAATTTTTGATATTTACAACGACATCTAAATAATTGAATT AAGTCAATTTGTTTAGATTCATCGAGATAGTCATTTTAAAAAAATGATTCATTGAA ATGTCTTAAAATACACATCAAAACAAACAATCTATACAATAGGAATTATATACT	5
M.synoviae	TCCTTACGGAGTACATTAATTTTACAAAAGGCATTTTTATTAACTGAAAGCTTTTAG AGAAAATTCTAAAAGCGGTTGTGTATCGCTTTTTTTGCCTTGGGCTATTGTATTTA GTTTTGAGAGAACAACCTCTCTTAAAATTGTTCTTTGAAAACTAAATAGTAATAAA GATATTACAACGACATCAAAAATATAAATTAATTAAGGTTAATTTGTTTTGATACCG AGTTTAAATTATTGAATAATAATTTATTAAAATGTCTTTGAATACATCATAACAATA TAACAATAGGACATATTGATACTAACTTTTAAAAAAGT	6

【Table 2】

Genus	Probe	Sequence	SEQ ID NO.
Mycoplasma	MP-CP1	TTCTTTGAAAACTGA	7
	MP-CP2	RWTCTTTVAAAACTRRATWN	8
M. arginini, M. arthritidis *M. cloacale, M. falconis* *M. faucium, M. hominis* *M. hyosynoviae,* *M. orale* *M. salivarium*	MP-CA1	MWTYGTRTCCAGTTTTGAGAG	9
	MP-CA2	TTTAGATCAACCTATAGAATA	10
M. bovis, M. fermentans *M. opalescens,* *M. primatum,* *M. spermatophilum,* *M. synoviae*	MP-CB1	RTATYTAGTTTTGAGAGRRCA	11
	MP-CB2	WWTRATTYATTRAAATGTCTT	12
	MP-CB3	GGKYAATTTGTTTWGAT	13
	MP-CB4	RATATTTACAMCGMCAYC	14
M. muris, M. penetrans *U. ureslyticum*	MP-CC1	CCTCCTTTCTATCGGAGTAMA	15
	MP-CC2	CGGATTCTATTTAGTTTTGAG	16
M. neurolyticum, *M. pulmonis*	MP-CD1	TAAAATAGATACCTTAAKATA	17
	MP-CD2	GTATYYAGTTTTGAAAG	18
	MP-CD3	CTTGCCAAWTAGWTWT	19
M. genitalium, *M. pirum* *M. penumoniae*	MP-CE1	AWACRACAATCTTTCTAGTTC	20
	MP-CE2	AATAAGTTACTAAGGGCTTAT	21
Acholeplasma	AP-CP1	TCATCATATTCAGTTTTG	22
	AP-CA1	GGGCCTRTAGCTCAGYTGGTT	23
	AP-CA2	AGAGCRCWCGCYTGATAAGCG	24
	AP-CA3	WGRGGTCGATGGTTCRAGTCC	25
	AP-CB1	TCATCATATTCAGTTTTGARR	26
	AP-CB2	AGTCTTTGAAAAGTAGATAAA	27

【Table 3】

Species	Probe	Sequence	SEQ ID NO.
M. arginini	MP-arg1	AGATTATATCATACAATAGA	28
	MP-arg2	GAGTACATAAATGTTATGGAA	29
M. arthritidis-faucium	MP-arf1	TGAAGCCCGATGGTGGCTTCG	30
	MP-arf2	TGAGAGAACTAAACTTCTCTC	31
	MP-arf3	GAATACAAAATCAATACAATA	32
M. fermentans	MP-fer1	ATGTACTATTAACTTATTTCAC	33
	MP-fer2	TACAAAAGAGTACTTTTTAAA	34
	MP-fer3	TTTTTATGGGTCTAAAGCTTT	35
	MP-fer4	GAACAATATTTTTTTCTCTCA	36
	MP-fer5	ATAACAAACTATAACAATAGG	37
M. hominis	MP-hom1	ATTTATCTCTCGGTTCTTT	38
	MP-hom2	ATATTTATATTTTATAAGACA	39
	MP-hom3	ATTGATATATTAATTAATATT	40
M. hyorhinis	MP-hyo1	GAATAGCAAATAACAATATGATT	41
	MP-hyo2	CGGAGTACATTAGTCTTAATT	42
	MP-hyo3	TTACATAATCGATTCGTGTCT	43
	MP-hyo4	AGCTTTAAGTTCTCAATTATA	44
	MP-hyo5	TTCATATTTATTATTTCAACG	45
	MP-hyo6	AACGATCTTTTTTATAACCGA	46
	MP-hyo7	TTAAATTTCTAAAATAGATTA	47
	MP-hyo8	AGATATTTATCTTTAGCAATA	48
M. neurolyticum	MP-neu1	GGTTATTATGGGCTTGCTA	49
	MP-neu2	GGTTATTTAAAAATCCTTTTA	50
	MP-neu3	TAATTTTTTCTTTCTAATTAA	51
M. opalescens	MP-opa1	CATCATAATGTAACCAATAC	52
	MP-opa2	ACAAAAATCATTATTTTTAAT	53
	MP-opa3	TTTAATGATTATTAACCTTTT	54
	MP-opa4	TTATGTGCTTTGTTTTTATGG	55
	MP-opa5	TATGGTCTACAAAGCTTATAT	56
	MP-opa6	GATAAAAAACAATCATAAATT	57
M. orale	MP-ora1	CATAAATAGTTAATGGCTCA	58
	MP-ora2	ATAGAGACAAATACAAAAACA	59
	MP-ora3	GGTCAAAAATACTTATACGTA	60
M. pirum	MP-pir1	TAGTTCTTTGTGTGAATAACA	61
	MP-pir2	CTTTATACACCTTATTACAAT	62
	MP-pir3	TAAAATCCAATTTAAATGTTA	63
	MP-pir4	GCAAATTTGATGTCAACATTT	64
	MP-pir5	AATTAATCTCTCCTATTACTT	65

	MP-pir6	TTAAAGTAGTAGAGATGGTTC	66
	MP-pir7	CAAATATCAAATGCTAATGGA	67
	MP-pir8	ATGCTAATGGATATCAAAAAA	68
M. penetrans	MP-pen1	AAGAGTAAGTTCTAGGTCG	69
	MP-pen2	CATTAAAGCTAAGTAACAAAT	70
	MP-pen3	TCCTAAACTGAAATTTATCT	71
	MP-pen4	TTATATAAGAGTAAGTTCTAG	72
	MP-pen5	ATTTTTCTCTCAAGATAGTTC	73
	MP-pen6	TCTAATCATACTTGTTATTTT	74
M. pulmonis	MP-pul1	AATTTTTGATCCGAGTCATT	75
	MP-pul2	CATTTTTCTATCAATAGTTAT	76
	MP-pul3	TATGTGTATCTTGCCAATTAG	77
	MP-pul4	TTCTATCTTTCAAAACAAATA	78
	MP-pul5	TATAAATTAATATGATAACGT	79
	MP-pul6	TCATCAAAATGTAAAATTTTT	80
	MP-pul7	AAAAATAAAATAGATACCTTA	81
	MP-pul8	AAATAAATTTCAACAATAGGA	82
M. salivarium	MP-sal1	TAATGGATTTAATTTTCGTG	83
	MP-sal2	TATCAAATCAATATAATATTT	84
M. cloacale	MP-clo1	AGTACAATTCTCACTGTTATG	85
	MP-clo2	TAGAATATTCAAGACATATAC	86
M. falconis	MP-fal1	GAGTACAACTTCTGTTATG	87
	MP-fal2	AGAATACAAAAATATAGACAA	88
	MP-fal3	ATTGAAAAATTATTAATTAAT	89
M. hyosynoviae	MP-hyos1	CTAGACTAAAGTTAATGGTAC	90
	MP-hyos2	AATTATCAAATTAATATTTCA	91
M. muris	MP-mur1	TATAGAAAACCCCCACATCA	92
	MP-mur2	TATTAGAATATTTTAAATATT	93
	MP-mur3	GATTATTACACCATATTAGAA	94
	MP-mui4	TCAATAAACCTAAATAAAAAA	95
M. primatum	MP-pri1	GTAGACATAACCCCAGCTA	96
	MP-pri2	CAAACGTCTATCGCTTTTTAG	97
	MP-pri3	TCATGGGCTTTTAATAGGGTC	98
	MP-pri4	ACCCCAACTCCCATCAAAAAT	99
M. spermatophilum	MP-spe1	TTCATCGAGATAGTCATTTTA	100
	MP-spe2	CAAACATACATTCAAATTTT	101
	MP-spe3	TTTTGACTGAATGTTATTAAC	102
	MP-spe4	TTTGTTATGTGACTTTTATGG	103
	MP-spe5	AAAACAAACAATCTATACAAT	104

M. synoviae	MP-syn1	TTGGCTTGGGCTATTGTATT	105
	MP-syn2	GCGGTTGTGTATCGCTTTTTT	106
	MP-syn3	ACCTCTCTTAAAATTGTTCTT	107
	MP-syn4	CCGAGTTTAAATTATTGAATA	108
	MP-syn5	CATCATAACAACATAACAATA	109
M. pneumoniae	MP-pne1	GTAAATTAAACCCAAATCCC	110
	MP-pne2	ATCTTTAATAAAGATAAATAC	111
	MP-pne3	CTAAACAAAACATCAAAATCC	112
	MP-pne4	AAAGAACATTTCCGCTTCTTT	113
M. genitalium	MP-gen1	CACCCCTTAATTTTTTCGG	114
	MP-gen2	AATGGAGTTTTTATTTTTTATTTA	115
	MP-gen3	CCCAAATCAATGTTTGGTCTC	116
	MP-gen4	CAACTAACACACTTGGTCAGT	117
	MP-gen5	AGAATGTTTTTGAACAGTTC	118
	MP-gen6	TAGTTCCAAAAATAAATACCA	119
M. bovis	MP-bov1	TATAACCAAAATTAAAAGACCTA	120
	MP-bov2	GTCATGGCTTTTATTAATAGG	121
U. urealyticum	UP-ure1	CATTAAGTTGTCAGTGAA	122
	UP-ure2	TAATTTACGTACTAATAAGTG	123
	UP-ure3	TTTATTAAAATCCATATGAAT	124
	UP-ure4	AAGCCACTTTTTTAAAAATTT	125
	UP-ure5	CCATAATAATTAATTTATTAT	126
	UP-ure6	ATTATCAACAAATCTTTCTAA	127
A. laidlawii	AP-lai1	AACACTTAGCACAAGATGAC	128
	AP-lai2	CTTTCTAAGGAGAAAGGCTAA	129
	AP-lai3	ATGACTACTAGTAAGTAGTAA	130
	AP-lai4	GTAGTAATATTCTCTAAATTT	131
	AP-lai5	TTAAAGTAATTTAAGTGTTTC	132
	AP-lai6	TAAATGATGTCTGAAAAGAAA	133

* Mixed Base의 Code Name

M : A + C, W : A + T, Y : C + T, R : A + G

K : G + T, V : G + A + C, N : A + G + C + T

Brief Description of the Drawings

FIGS. 1a to 1f show multiple sequence alignments of each ITS region of *Mycoplasma* and *Ureaplasma* for selecting genus-specific probes.

FIGS. 2a to 2c show multiple sequence alignments of each ITS region of *Acholeplasma* for selecting genus-specific probes.

FIG. 3 shows a result of PCR amplification using primer pairs which can amplify ITS target sequences of many *Mycoplasma* and its related strains

FIG. 4 shows a microarray comprising probes for detecting genotypes of *Mycoplasma* and its related strains as a set on a support.

FIGS. 5a to 5k show results of image analysis of specific hybridization reaction of each probes for detecting genotypes of *Mycoplasma* and its related strains and results of numerical analysis calculated from pixel intensity.

Best mode for carrying out the Invention

The present invention will be decribed in greater detail by means of following examples. The following examples are for illustrative purpose and are not intended to limit the scope of the invention.

Example 1: Incubation of *Mycoplasma* and its related strains and Isolation of Genomic DNA

Total 25 kinds of strains, including 1 kind of *Acholeplasma*, 23 kinds of *Mycoplasma*, and 1 kind of *Ureaplasma* were obtained from the American Type Culture Collection (ATCC). The strains were cultured in each culturing media under each culturing conditions according to manual provided by ATCC. From the cultured media, strain colonies were obtained with a white gold ear and input in 1.5㎖ tube, 100㎕ of InstaGene matrix (Bio-Rad, USA) was added thereto and suspended, and reaction was performed at 56℃ for 30 minutes in constant temperature bath. And then, the reactant was shook for 10 seconds, heated at 100℃ for 8 min, shook again for 10 sec, centrifuged at 12,000 rpm for 3 min, transferred to new tube, and freeze-stored at -20℃. The product was used as template DNA of PCR reaction.

The strains used were as followed:

Acholeplasma laidlawii (ATCC 25937)
Mycoplasma arginini (ATCC 23838)
Mycoplasma arthritidis (ATCC 19611)
Mycoplasma bovis (ATCC 27368)
Mycoplasma cloacale (ATCC 35276)

Mycoplasma falconis (ATCC 51372)
Mycoplasma faucium (ATCC 25293)
Mycoplasma fermentans (ATCC 19989)
Mycoplasma genitalium (ATCC 33530)
Mycoplasma hominis (ATCC 23114)
Mycoplasma hyorhinis (ATCC 17981)
Mycoplasma hyosynoviae (ATCC 25591)
Mycoplasma muris (ATCC 33757)
Mycoplasma neurolyticum (ATCC 19988)
Mycoplasma opalescens (ATCC 27921)
Mycoplasma orale (ATCC 23714)
Mycoplasma penetrans (ATCC 55252)
Mycoplasma pirum (ATCC 25960)
Mycoplasma pneumoniae (ATCC 15531)
Mycoplasma primatum (ATCC 15497)
Mycoplasma pulmonis (ATCC 14267)
Mycoplasma salivarium (ATCC 23064)
Mycoplasma spermatophilum (ATCC 49695)
Mycoplasma synoviae (ATCC 25204)
Ureaplasma urealyticum (ATCC 27618)

Example 2: Preparation of probes for detection of *Mycoplasma* and its related strains

The probes used for detection of *Mycoplasma* and its related strains were selected based on a result of multiple alignment of ITS sequences of *Mycoplasma*, *Acholeplasma* and *Ureaplasma*. Among *Mycoplasma* and its related species, 16S rRNA sequences has high similarity of 74~97%, whereas ITS sequences has lower similarity of 25.4~78.8% except for between *M. salivarium* and *M. hyosynoviae*, and *M. hominis* and *M. falconis*. In other words, ITS contains a region more polymorphic than 16S rRNA which is useful for designing probes for

detection of *Mycoplasma* and its related strains. However, to complement specificity between *M.salivarium* and *M. hyosynoviae,* and *M. hominis* and *M. falconis* having a high ITS similarity, more restrictive and strict probes were designed.

In the present invention, the oligonucleotide probes for detection of *Mycoplasma* and its related strains were prepared by synthesizing 15-25 bases of specific probe with 15 bases of dT spacer at 5' end. Probes for detection of *Mycoplasma* and its related strains are not restricted to the sequences disclosed in Tables 2 and 3 and any primer and probes comprising the sequences can be used in the present invention.

1. Preparation of probes for detection of *Mycoplasma* and *Ureaplasma*

① Preparation of probes for genus-specific detection of *Mycoplasma* and *Ureaplasma*

For genus-specific hybridization with all *Mycoplasma* and Ureaplasma genus, probes of SEQ ID Nos. 7 and 8 in Table 2 were designed from conserved sequences of ITS of *Mycoplasma*. Further, each Group-based conserved sequences targeted to *Mycoplasma* ITS were designed as follows. For detecting Group Ⅰ(*M. arginins. M. arthritidis, M. cloacale, M. falconis, M. faucium, M. hominis, M. hyosynoviae, M. orale, M. salivarium*), probes of SEQ ID Nos. 9 and 10 were designed. For detecting Group Ⅱ(*M. bovis. M. fermentans, M. opalescens, M. primatum, M. spermatophilum, M. synoviae*), probes of SEQ ID Nos. 11, 12, 13 and 14 were designed. For detecting Group Ⅲ(*M. muris, M. penetrans, U. urealyticum*), probes of SEQ ID Nos. 15 and 16 were designed. For detecting Group Ⅳ(*M. neurolyticum, M. pulmonis*), probes of SEQ ID Nos. 17, 18 and 19 were designed. For detecting Group Ⅴ(*M. genitalium, M. pirum, M. pneumoniae*), probes of

SEQ ID Nos. 20 and 21 were designed.

② Preparation of probes for species-specific detection of *Mycoplasma* and *Ureaplasma*

For species-specific hybridization with each *Mycoplasma* and *Ureaplasma* species, 100 kind of probes of SEQ ID Nos. 28 to 127 in Table 3 were designed from species-specific sequences of ITS of *Mycoplasma* and *Ureaplasma,* which can detect 25 kind of *Mycoplasma* strains.

2. Preparation of probes for detection *Acholeplasma*

① Preparation of genus-specific probes for detection *Acholeplasma*

For genus-specific hybridization with all *Acholeplasma* genus, probes of SEQ ID No. 22 in Table 2 was designed from conserved sequences targeted to both of ITS1 and ITS2 of *Acholeplasma.* Further, each Group-based conserved sequences targeted to each *Acholeplasma* ITS1 and ITS2 were designed as follows. For Group Ⅰ targeted to ITS1, probes of SEQ ID Nos. 23, 24 and 25 were designed. For Group Ⅱ targeted to ITS2, probes of SEQ ID Nos. 26 and 27 were designed.

② Preparation of species-specific probes for detection *Acholeplasma*

For species-specific hybridization with each *Acholeplasma* species, probes of SEQ ID Nos. 128 to 133 in Table 3 were designed from species-specific sequences of ITS of Acholeplasma.

Example 3: Preparation of target DNA

1. Preparation of target DNA for detection of *Mycoplasma* and its

related strains

For preparing target DNA for detection of *Mycoplasma* and its related strains, 187~290bp size of ITS regions were selectively amplified using 5'biotin-GTG(C/G)GG(A/C)TGGATCACCTCCT-3' (MP16SF-2) and 5'-biotin-GCATCCACCA(A/T)A(A/T)AC(C/T)CTT-3' (MP23SR-2), and 5'-biotin-AAAGTGGGCAATACCCAACGC-3' (M78) and 5'-biotin-CCACTGTGTGCCCTTTGTTCCT-3' (R34) which were biotin-labeled respectively (Tang et al., 2000.). To prepare genomic DNAs of *Mycoplasma* and its related strains isolated in Example 1, PCR were carried out using the above primers in the following conditions: denaturation at 94℃ for 3 minutes, 30 cycles of amplification at 94℃ for 30 seconds, at 55℃ for 2 minutes and at 72℃ for 2 miniutes, and final extension at 72℃ for 10 minutes. After the reaction, the reaction products were analyzed by ELECTROPHORESIS on a 2% agarose gel. FIG. 3 is an electrophoresis image taken after the PCR performed using primers capable to amplify ITS target sequences of several *Mycoplasma*.

Example 4: Probe immobilization on support

Among the probes prepared in Example 2, each representative probes for *Mycoplasma*, Acholeplasma and Ureaplasma were selected. Each of the selected probes was transferred to 384-well microplate, diluted to a concentration of 50 pmole by adding spotting solution, and immobilized on a slide glass using a microarrayer (Cartesian Technologies, USA). In FIG 4, each probes for detection of *Mycoplasma* and its related strains correspond to SEQ ID Nos. 7, 28, 30, 33, 38, 41, 49, 52, 58, 61, 69, 75, 83, 85, 87, 30, 90, 92, 96, 100, 105, 110, 114, 120, 122, 22, 128, and 7 in order. Two spots of each kind of the probes were attached to the support and left in a slide box at room temperature for 24 hours or in a dry oven at 50℃ for about 5 hours to be fixed to the surface of the support.

Example 5: Unimmobilized probe washing

The slide glass after the process in Example 4 was washed with a 0.2% SDS buffer solution and then distilled water at room temperature to remove unimmobilized probes. The washed slide glass was immersed in a sodium borohydride ($NaBH_4$) solution for 5 minutes and then washed again at 100℃. Final washing with a 0.2% SDS solution and then distilled water was followed by centrifugation to fully dry the slide glass.

Example 6: Hybridization

The biotin-labeled target products prepared in Example 3 were thermally treated to be denaturated into single strands and cooled to 4℃. A hybridization reaction solution containing 2㎕ of the target products was prepared. This hybridization reaction solution was portioned on the slide glass after the process in Examples 4 and 5, and the slide glass was covered with a cover slip and reacted at 25℃ for 1 hours.

Example 7: Unhybridized tart DNA washing

TO WASH OUT UNHYBRIDIZED TARGET DNAS, THE COVER SLIP WAS REMOVED USING A 2X SSC WASHING SOLUTION (300MM NACL, 30MM NA-CITRATE, PH 7.0), AND THE SLIDE WAS WASHED WITH 2X SSC AND THEN 0.2X SSC, FOLLOWED BY CENTRIFUGATION TO FULLY DRY THE SLIDE GLASS.

Example 8: Staining and Result analysis

To determine hybridization of PCR products and probes, Cy5-streptavidin or Cy3-streptavidin (Amersham pharmacia biotech, USA) was diluted with 6x SSC and BSA (Bovine Serum Albumin), about 40㎕ of dilutes was portioned on slide glass, and the slide glass was covered

with a cover slip to block light and reacted at 50°C for about 20 minutes. After the reaction, the cover slip was removed using a 2X SSC solution, and the slide was washed with 2X SSC and then 0.2X SSC. The hybridized result was scanned using a non-confocal laser scanner (GenePix 4000A, Axon Instruments, U.S.A.) and analyzed by image analysis.

FIG. 5 shows results of image analysis of specific hybridization reaction of each probes for detecting genotypes of representative 11 kinds of *Mycoplasma* and its related strains and results of numerical analysis calculated from pixel intensity.

FIG. 5a shows results of hybridization reaction of genus-specific probe (SEQ ID No. 7) and species-specific probe (SEQ ID No. 85) of *M. cloacale*. FIG. 5b shows results of hybridization reaction of genus-specific probe (SEQ ID No. 7) and species-specific probe (SEQ ID No. 87) of *M. falconis*. FIG. 5c shows results of hybridization reaction of genus-specific probe (SEQ ID No. 7) and species-specific probe (SEQ ID No. 90) of *M. hyosynoviae*. FIG. 5d shows results of hybridization reaction of genus-specific probe (SEQ ID No. 7) and species-specific probe (SEQ ID No. 49) of *M. neurolyticum*. FIG. 5e shows results of hybridization reaction of genus-specific probe (SEQ ID No. 7) and species-specific probe (SEQ ID No. 52) of *M. opalescens*. FIG. 5f shows results of hybridization reaction of genus-specific probe (SEQ ID No. 7) and species-specific probe (SEQ ID No. 69) of *M. penetrans*. FIG. 5g shows results of hybridization reaction of genus-specific probe (SEQ ID No. 7) and species-specific probe (SEQ ID No. 61) of *M. pirum*. FIG. 5h shows results of hybridization reaction of genus-specific probe (SEQ ID No. 7) and species-specific probe (SEQ ID No. 83) of *M. salivarium*. FIG. 5i shows results of hybridization reaction of genus-specific probe (SEQ ID No. 7) and species-specific probe (SEQ ID No.

100) of *M. spermatophilum*. FIG. 5j shows results of hybridization reaction of genus-specific probe (SEQ ID No. 7) and species-specific probe (SEQ ID No. 122) of *U. urealyticum*. FIG. 5k shows results of hybridization reaction of genus-specific probe (SEQ ID No. 22) and species-specific probe (SEQ ID No. 128) of *A. laidlawii*.

Industrial Applicability

As described above, the present invention provides a rapid and accurate assay method capable of simultaneously detecting many *Mycoplasma* and its related strains from a single sample using a microarray comprising novel oligonucleotides for detecting *Mycoplasma* and its related strains which are known as a source of contamination of cell lines and biological products and human pathogenic.

Also, the present invention provides an objective and credible assay method capable of tracing a contamination source for preventing expansion of infective *Mycoplasma* and its related strains and controlling a contamination of *Mycoplasma* against biological products and stem cells or cord blood cells which are useful for gene therapy and cell therapy.

Further, the present invention provides very specific and sensitive hybridization assay for detecting *Mycoplasma* and its related strains using oligonucleotide probes designed based on sequence analysis of ITS region of many *Mycoplasma* Strains.

[References]

1. Doblhoff-Dier O, Collins CH. (2001) Biosafety: future priorities for research in health care. J Biotechnol. 85 : 227-39.

2. Jung H, Wang SY, Yang IW, Hsueh DW, Yang WJ, Wang TH, Wang HS. (2003) Detection and treatment of *Mycoplasma* contamination in cultured cells. Chang Gung Med J. 26 : 250-8.

3. Wisher M. (2002) Biosafety and product release testing issues

relevant to replication-competent oncolytic viruses, Review. Cancer Gene Ther. 9 : 1056-61.

4. Hopert A, Uphoff CC, Wirth H, Hauser H, Drexler HG. (1993) Specificity and sensitivity of polymerase chain reaction in complarision with other methods for the detection of *Mycoplasma* contamination in cell lines. J Immunol Methods. 164 : 91-100.

5. Kong F, James G, Gordon S, Zelyski A, Gilbert GL. (2001) Species-Specific PCR for Identification of Common Contaminant Mollicutes in Cell Culture. Appl Environ Microbiol. 67 : 3195-200.

6. Dorigo-zetsma J.W., Zaat SAJ, Wertheim-van D, Spanjaard PME, Rijntjes J, Waveren V, Jensen JS, Angulo AF, Dankert J. (1997) Comparision of PCR, culture, and serological tests for diagnosis of *Mycoplasma* pneumoniae respiratory tract infection in children. J Clin Microbiol. 37 : 14-7.

7. Jensen JS, Borre MB, Dohn B. (2003) Detection of *Mycoplasma genitalium* by PCR Amplification of the 16S rRNA Gene. J Clin Microbiol. 41 : 261-266.

8. Uphoff CC, Drexler HG. (2002) ComparativePCR analysis for detection of *Mycoplasma* infections in continuous cell lines. In Vitro Cell Dev Anim. 38 : 79-85.

9. Gohlman HW, Weiner J 3rd, Schon A, Herrmann R. (2000) Identification of a small RNA within the pdh gene cluster of *Mycoplasma* pneumoniae and Mycoplasma genitalium. J Bacteriol. 182 : 3281-4.

10. Tang J, Hu M, Lee S, Roblin R. (2000) A polymerase chain reaction based method for detectiong *Mycoplasma/Acholeplasma* contaminants in cell culture. J Microbiol Methods. 39 : 121-6.

What is claimed is:

1. An ITS (Internal transcribed spacer) target DNA for genotyping *Mycoplasma* strains, comprising any one sequence selected from SEQ ID Nos. 1 to 6.

2. An oligonucleotide for genus-specific genotyping of *Mycoplasma* and *Ureaplasma* strains, comprising any one sequence selected from SEQ ID Nos. 7 to 21 or its complementary sequence.

3. An oligonucleotide for species-specific genotyping of *Mycoplasma* and *Ureaplasma* strains, comprising any one sequence selected from SEQ ID Nos. 28 to 127 or its complementary sequence.

4. An oligonucleotide for genus-specific genotyping of *Acholeplasma* strains, comprising any one sequence selected from SEQ ID Nos. 22 to 27 or its complementary sequence.

5. An oligonucleotide for species-specific genotyping of *Acholeplasma* strains, comprising any one sequence selected from SEQ ID Nos. 128 to 133 or its complementary sequence.

6. A microarray comprising more than one oligonucleotide selected from genus-specific and species-specific oligonucleotides for genotyping *Mycoplasma*, *Acholeplasma* and *Ureaplasma* strains according to any one from claims 2 to 5 as probes attached on a support.

7. The microarray according to claim 6, wherein the probes are any one selected from a group consisting of DNA, RNA, PNA, LNA and HNA.

8. The microarray according to claim 6, wherein the support is any

one selected from a group consisting of slide glass, plastic, membrane, semiconductive chip, silicon and gel.

9. A method for detecting *Mycoplasma* strains, comprising the following steps:

a) extracting nucleic acids from a sample;

b) amplifying target DNA among the extracted nucleic acids;

c) hybridizing the amplified target DNA with probes of the microarray according to claim 6; and

d) detecting signals generated from the hybridization reaction.

10. A kit for diagnosing *Mycoplasma* infection, comprising more than one oligonucleotide selected from genus-specific and species-specific oligonucleotides for genotyping *Acholeplasma*, *Mycoplasma* and *Ureaplasma* strains according to any one from claims 2 to 5.

Abstract of the Invention

The present invention relates to a method for detecting *Mycoplasma* and its related strains which are source of contamination of cell lines and biological products and human pathogenic. More particularly, the present invention relates to genus-specific and species-specific oligonucleotides for genotyping of *Mycoplasma*, *Acholeplasm* and *Ureaplasma* strains, microarray comprising the oligonucleotides, and method for detection of species using the microarray.

As described above, the present invention provides a rapid and accurate assay method capable of simultaneously detecting many *Mycoplasma* and its related strains from a single sample using a microarray comprising novel oligonucleotides for detecting *Mycoplasma* and its related strains which are known as a source of contamination of cell lines and biological products and human pathogenic. Further, the present invention provides an objective and credible assay method capable of tracing a contamination source for preventing expansion of infective *Mycoplasma* and its related strains and controlling a contamination of *Mycoplasma* against biological products and stem cells or cord blood cells which are useful for gene therapy and cell therapy.

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RO/KR 14.05.2005

FIG. 1a

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M. bovis            ------ATA------------------TGTTCTTTGAAAACTGAATAGTAAAATATTTTT   142
M. primatum         ------TT-------------------TGTTCTTTGAAAACTGAATAGTAAAATATTTTT   181
M. fermentans       ------ATT------------------TGTTCTTTGAAAACTGAATAGTAAA---TTTTT   177
M. lipofaciens      ------TT-------------------TGTTCTTTGAAAACTGAATAGTAAA---TTTTA   159
M. spermatophilum   ------AT-------------------TGTTCTTTGAAAACTGAATAGTAAA---TTTTT   196
M. synoviae         ------AAT------------------TGTTCTTTGAAAACTAAATAGTAA-------TAA   100
M. neurolyticum     TAATAAATGTTTT-----------AATATATTCTTTGAAAACTGAATAGCAAA---TA--T   176
M. pulmonis         -AACAAATA-------------------GTTCTTTAAAAACTGAATAGCATA---TAAAT   159
M. hyorhinis        ------ATA-------------------GTTCTTTGAAAACTGAATAGCAAA-----TAA   112
M. arthritidis      ------TT-------------------TGTTCTTTGAAAACTGAATA------------T   115
M. faucium          ------TT-------------------TGTTCTTTGAAAACTGAATA------------T   123
M. orale            ------TT-------------------TATTCTTTGAAAACTGAAT-------------T   108
M. hyosynoviae      ------TT-------------------TGTTCTTTGAAAACTGAAT-------------T   119
M. salivarium       ------TT-------------------TGTTCTTTGAAAACTGAAT-------------T   115
M. falconis         ------TT-------------------TGTTCTTTGAAAACTGAATA------------T    92
M. fusinis          ----------------------------GTTCTTTGAAAACTGAATA------------T    07
M. arginini         ------TT-------------------TGTTCTTTGAAAACTGAATA------------T    93
M. alkalescens      ------TT-------------------TGTTCTTTGAAAACTGAATA------------T    96
M. genitalium       CCAGTTCTGAAAG-AATGTTTTTGAACAGTTCTTTCAAAACTGAAAACGACA---------   160
M. pneumoniae       CCAGTTCTGAAAG-AACATTTCCGC-----TTCTTTCAAAACTGAAAACGACA---------   190
M. pirum            TAAATTTTTAAAGTAGTAGAGATGG-----TTCTTTGAAAACTGGATACAACA---------   213
M. muris            TT-------------------------------CTTTGAAAACTGAATATTTGTA---------   106
M. penetrans        TT-------------------------------CTTTGAAAACTGAATATTTATAA--------   184
U. urealyticum      TTAATTTATTATG---GATGATCGA------TCTTTGAAAACTGAATAAT-TAT--------   199
                                                  **** ******    *
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FIG. 1b

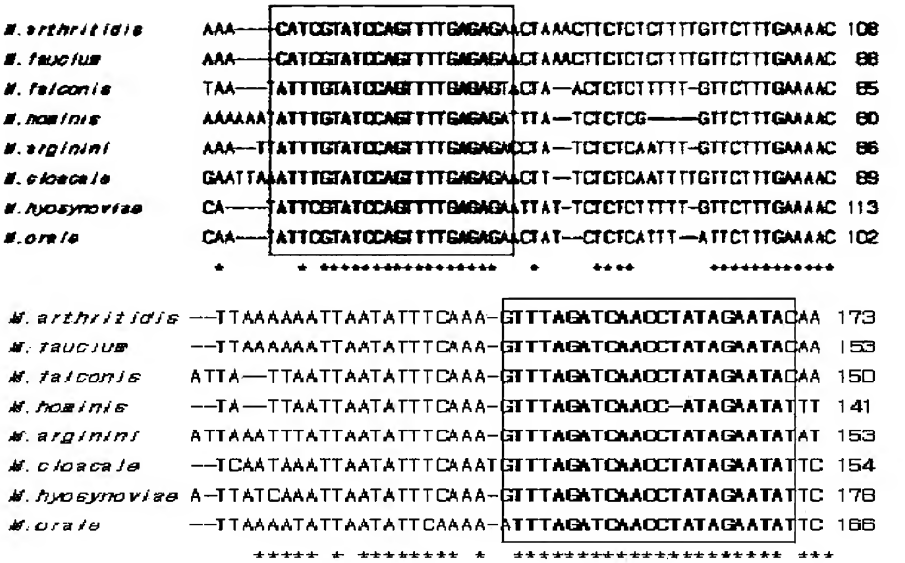
M. arthritidis
M. fauciun
M. falconis
M. hominis
M. arginini
M. cloacale
M. hyosynoviae
M. orale

FIG. 1c

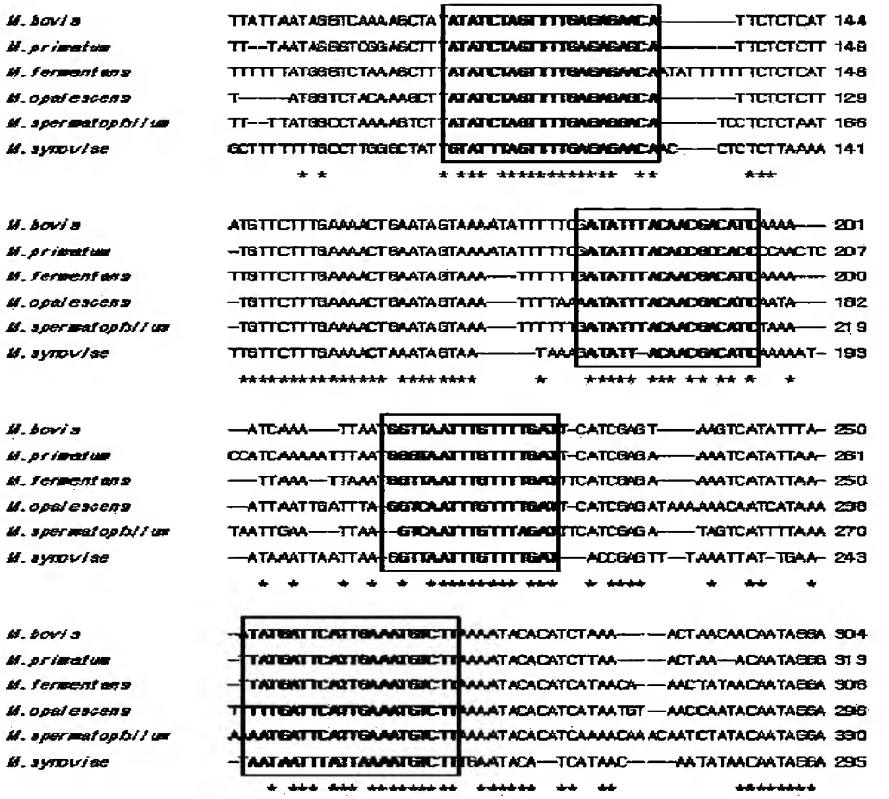
M. bovis
M. primatum
M. fermentans
M. opalescens
M. spermatophilum
M. synoviae

FIG. 1d

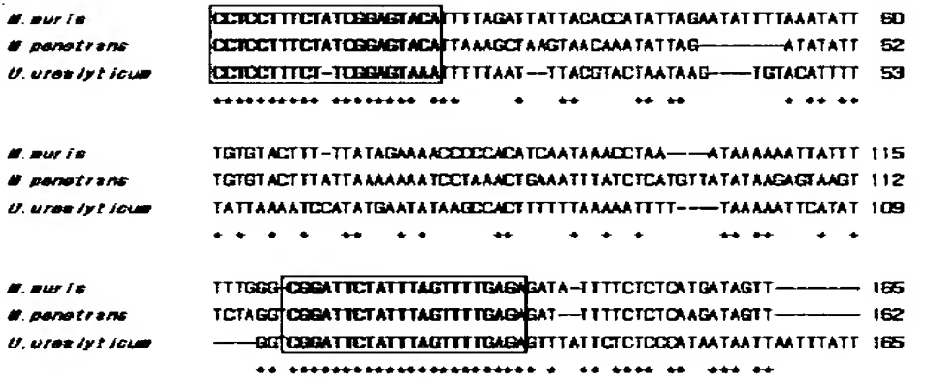
M. murfis
M. penetrans
U. urealyticum

FIG. 1e

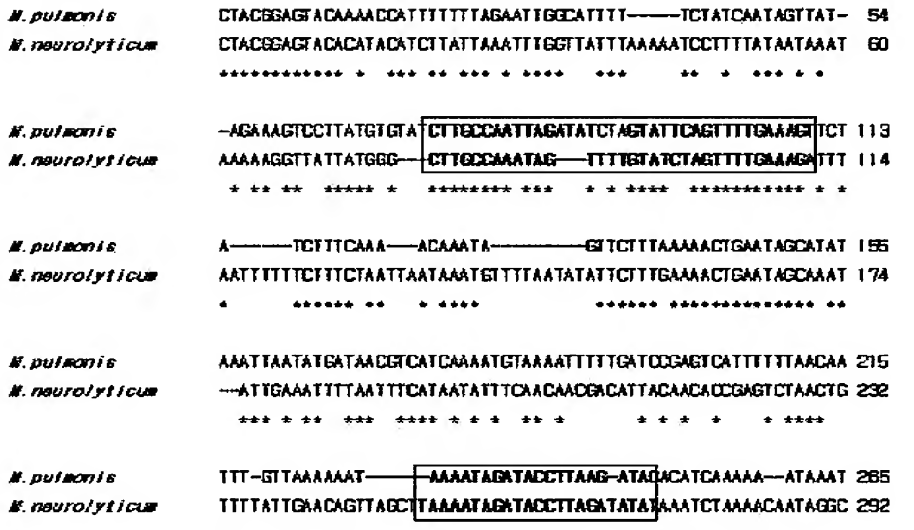
M. pulmonis
M. neurolyticum
M. pulmonis
M. neurolyticum
M. pulmonis
M. neurolyticum
M. pulmonis
M. neurolyticum

FIG. 1f

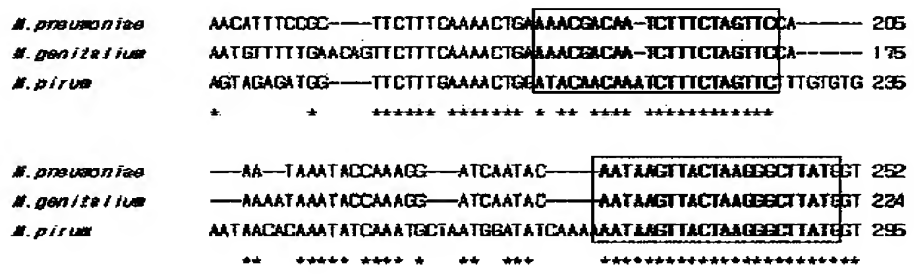
M. pneumoniae
M. genitalium
M. pirum
M. pneumoniae
M. genitalium
M. pirum

FIG. 2a

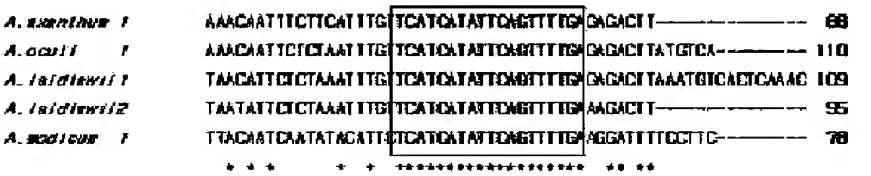

FIG. 2b

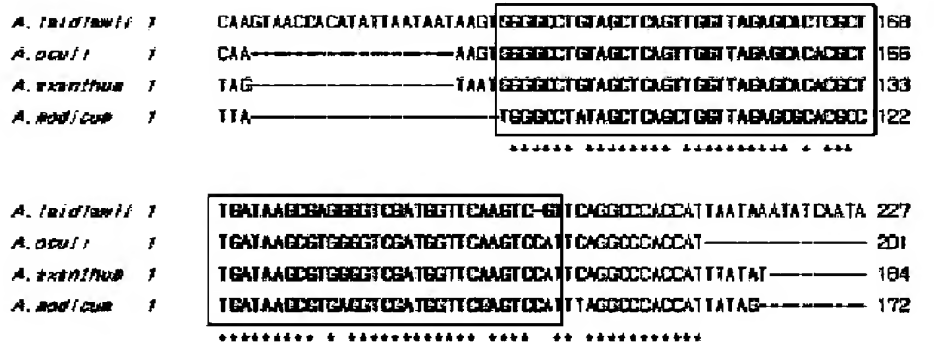

FIG. 2c

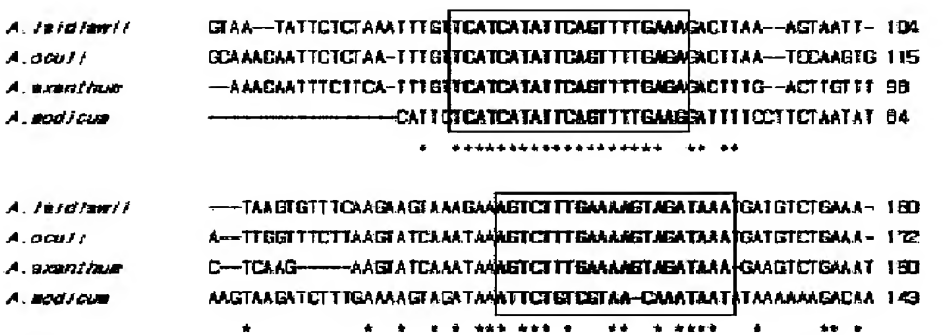

FIG. 3a

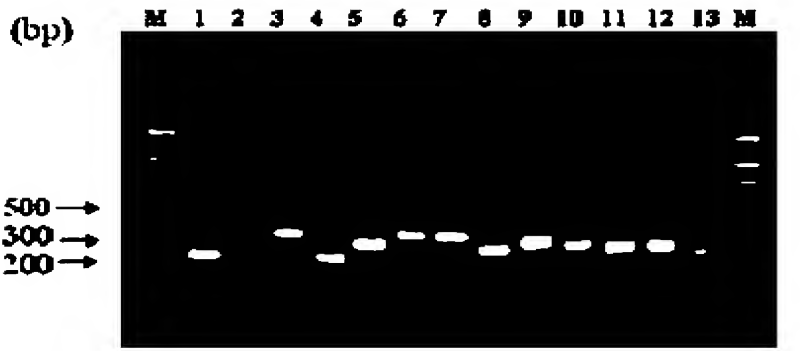
(bp)
M 1 2 3 4 5 6 7 8 9 10 11 12 13 M
500
300
200

FIG. 3b

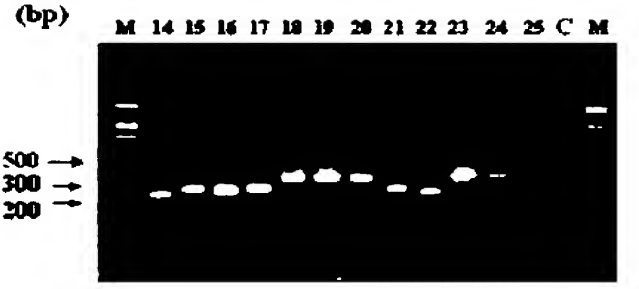
(bp)
M 14 15 16 17 18 19 20 21 22 23 24 25 C M
500
300
200

FIG. 4

MP-C [7]	*M. arginini* [28]	*M. arthritidis* [30]	*M. fermentans* [33]	*M. hominis* [38]	*M. hyorhinis* [41]
M. neurolyticum [49]	*M. opalescens* [52]	*M. orale* [58]	*M. pirum* [61]	*M. penetrans* [69]	*M. pulmonis* [75]
M. salivarium [83]	*M. cibacale* [85]	*M. falconis* [87]	*M. faucium* [30]	*M. hyosynoviae* [90]	*M. muris* [92]
M. primatum [96]	*M. spermatophilum* [100]	*M. synoviae* [105]	*M. pneumoniae* [110]	*M. genitalium* [114]	*M. bovis* [120]
U. urealyticum [122]			AP-C [22]	*A. laidlawii* [128]	MP-C [7]

*[ ] corresponds to SEQ ID No's of Tables 2 and 3.

FIG. 5a

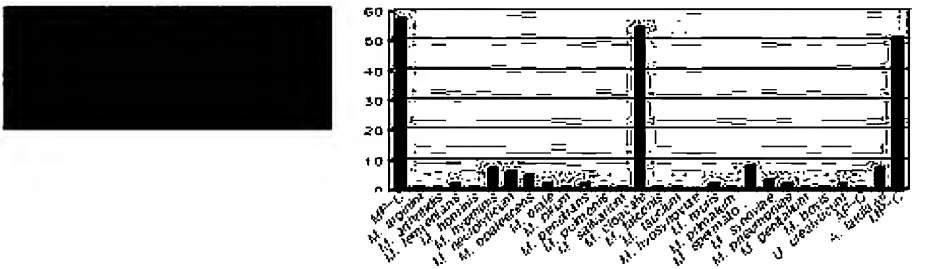

FIG. 5b

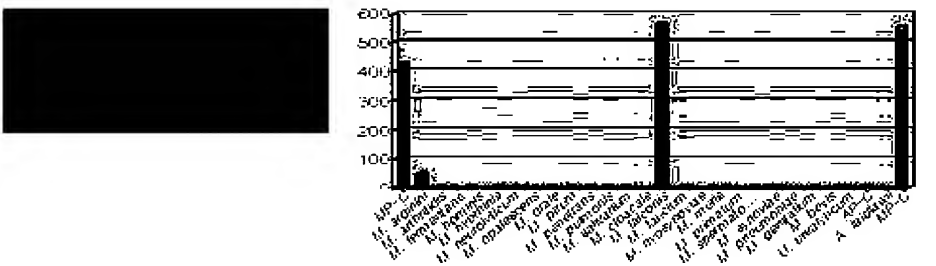

FIG. 5c

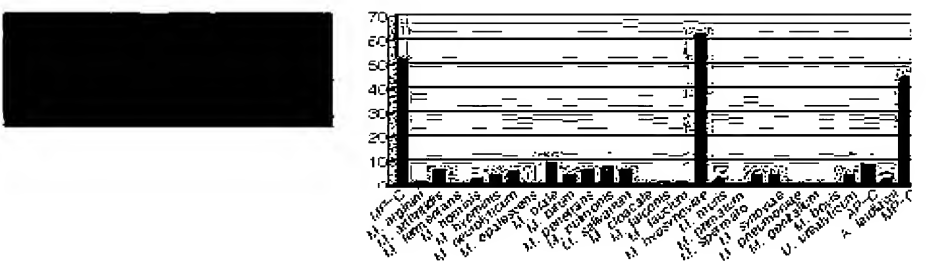

FIG. 5d

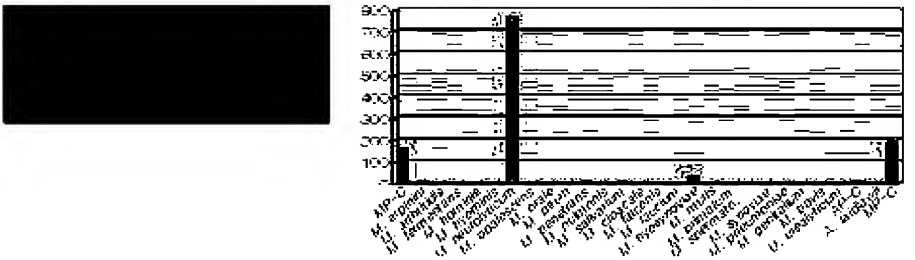

FIG. 5e

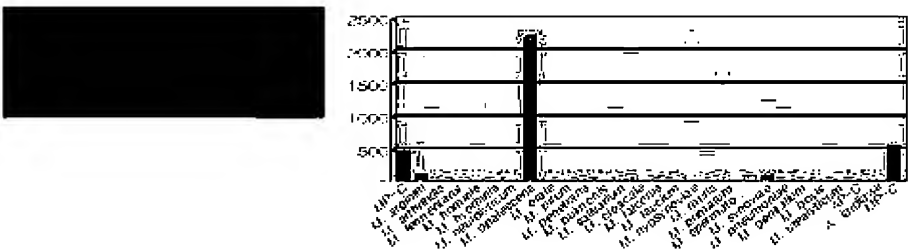

FIG. 5f

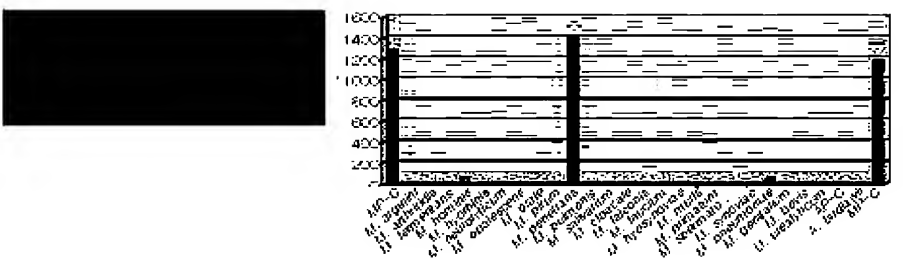

FIG. 5g

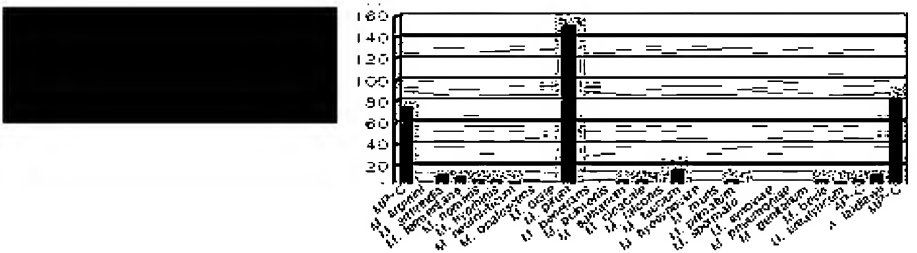

FIG. 5h

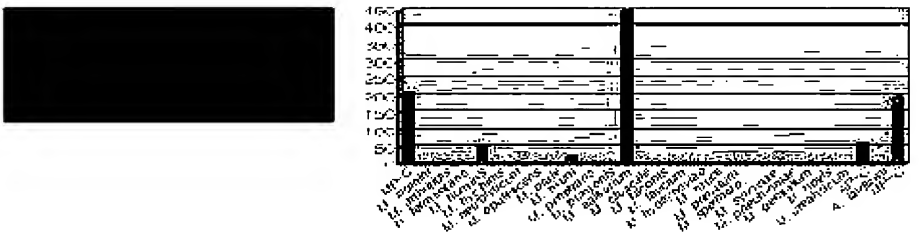

FIG. 5i

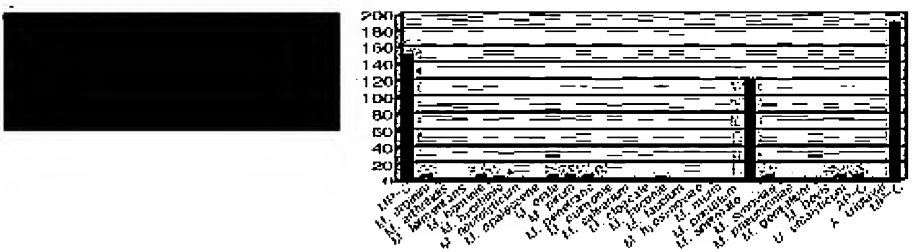

FIG. 5j

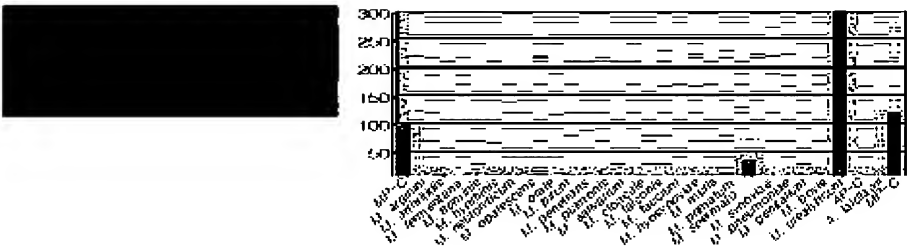

FIG. 5k